BIOTIN-BINDING RECEPTOR MOLECULES

Field of the Invention

This invention relates to membrane-spanning proteins having biotin-binding activity.

Background to the Invention

Biotin (vitamin H) is a readily water-soluble substance found at low concentrations in blood and tissues. The biological role of biotin is as a carrier of activated CO_2 and it permits the transfer of CO_2 to acceptors without the need for additional free energy. The activated carboxybiotin is usually attached to an enzyme that is required for the formation of carboxybiotin. For example, biotin may be attached to pyruvate carboxylase which, in the presence of acetyl CoA, catalyses the formation of carboxybiotin and the subsequent transfer of the activated carboxyl group to pyruvate, to form oxaloacetate.

Biotin also binds with one of the highest naturally known affinities to avidin, a 63 kDa glycoprotein from chicken egg white, and to streptavidin, a non-glycosylated protein from the bacterium *Streptomyces avidinii*. The binding is almost irreversible in character (Ka 10^{15} mol^{-1}). The affinity between avidin and biotin has proved very useful in a wide variety of bioanalytical applications. For example, the avidin-biotin complex has been used successfully in a wide variety of detection systems where target molecules are combined with biotin through its carboxy terminus, to form biotinylated molecules which may be easily detected or separated from solution. Biotinylation can occur without changing the biological or physiochemical properties of the various molecules and without affecting the binding capacity of the biotin prosthetic group to avidin.

Summary of the Invention

It has now been realised that the biotin-binding activity of avidin and streptavidin may be utilised in the production of transmembrane proteins capable of binding biotinylated molecules.

Proteins of the present invention may comprise a cytoplasmic domain, a membrane-spanning domain and an extracellular domain, wherein the extracellular domain comprises biotin-binding activity. The extracellular domain may comprise avidin or streptavidin functional activity.

Using proteins or nucleic acid molecules of this invention, it is possible to target biotinylated molecules to specific sites in tissues. Molecules targeted in this way may be taken up by the tissues or cells by endocytosis, allowing the molecules to exert their effects within or on the cell.

Description of the Drawings

Figure 1 is a schematic illustration of a fusion protein of the present invention, where A represents avidin and B represents the membrane-spanning domain of an endocytotic receptor (and C represents biotin);

Figure 2 is a schematic illustration of a cloning strategy using a shuttle vector; and

Figure 3 is a schematic illustration of a cloning strategy using a retrovirus vector.

Description of the Invention

Proteins of the present invention may be produced using conventional recombinant DNA technology. Typically, a DNA sequence coding for the functional domain of a biotin-binding protein such as avidin, streptavidin or a related protein, is engineered into a genetic construct which comprises a DNA sequence coding for a protein having membrane-spanning properties. Examples of avidin and streptavidin-related proteins include AVR-1-AVR-5, AVR-X-AVR-V, Stv1 and Stv2.

The individual domains of the fusion protein may be amplified by polymerase chain reaction or isolated from the parent cDNA using restriction enzyme digestion, isolation and purification, e.g. using gel electrophoresis, and subsequent ligation, e.g. using DNA ligase. The fusion protein construct may then be transfected into any suitable

host cell, cultured and isolated using standard protein purification techniques.

The construct may also be used as naked DNA or as a plasmid/liposome, plasmid/polyethyleneimine, plasmid/dendrimer or plasmid/peptide complex.

Alternatively, the construct may be introduced into a replication-deficient virus which can be used to target the construct to specific sites *in vivo*. For example, the construct may be a retroviral vector comprising the appropriate cDNA for the fusion protein. A replication-deficient retrovirus, e.g. Moloney murine retrovirus, may then be used for the stable transfection of target cells and tissues. Other viruses that can be used include replication-deficient adenoviruses, adeno-associated viruses, herpes viruses, papilloma viruses and sinibis viruses. Additional viruses will be apparent to those skilled in the art.

In addition to the functional domains of avidin, streptavidin or related protein, the fusion protein will typically comprise the membrane-spanning domains of endocytotic receptors. The use of these receptors enables the uptake of biotinylated molecules into a target cell. Suitable receptors that may be used in this invention include the scavenger receptor class A, low density lipoprotein receptor, very low density lipoprotein receptor, transferrin receptor and the LOX-1 receptor. The fusion protein may also comprise a linker between the receptor protein and the avidin peptide sequences. The linker may be any length, provided that the functional activity of the different components of the fusion protein is retained.

In general, the fusion between avidin or streptavidin peptide sequences and the receptor peptide sequences is between the extracellular domain of the receptor protein and any site outside of the biotin-binding site of avidin or streptavidin.

The following Example illustrates the invention.

Example

A DNA construct was created between the bovine scavenger receptor class A (ScR) (Kodama *et al.* (1990) Nature 343:531-535) and avidin (Green (1975) Adv. Prot. Chem. 29:85-133), which codes for a protein having a ScR cytoplasmic domain, membrane-spanning domain and α-helical coiled domain, ligated to a biotin-binding domain. The complete amino acid sequence of the fusion protein is shown in SEQ ID No 2 where amino acids 1-53 represent the cytoplasmic domain; amino acids 55-79 represent the transmembrane domain; amino acids 81-111 represent a spacer domain; and amino acids 113-272 represent the α-helical coiled domain. Amino acids 273-400 represent the mature avidin peptide sequence derived from avidin cDNA (Gope *et al.* (1987) Nucleic Acid Res. 15:3595-3606) lacking a secretion signal.

Briefly, the cDNA for ScR was obtained from cultured cells previously transfected with a plasmid (PLScRNL) containing the ScR cDNA with an internal Rous Sarcoma Virus promoter and *Hin*dIII restriction sites. The isolated cDNA was then inserted into a *Hin*dIII site of the retrovirus vector pLS1ARNL. The avidin cDNA was produced by the polymerase chain reaction and then inserted into the retrovirus vector at a Sty 1 restriction site on the ScR cDNA. The cDNA embodying the invention is shown as SEQ ID No 1, where nucleotides 1-989 represent a long terminal repeat from Mo-MuSV; nucleotides 1071-2270 represent the coding region for the fusion protein; nucleotides 2376-3101 represent an untranslated region from bovine scavenger receptor I cDNA; nucleotides 3107-3376 represent an RSV promoter region; nucleotides 3727-4522 represent a neo R gene; and nucleotides 4540-5177 represent a long terminal repeat from Mo-MuLV.

Figs. 2 and 3 refer to processes used in this Example. More specifically, Fig. 2 shows how the ScR cDNA with an internal RSV promoter was cut from plasmid pLScRNL by HindIII and cloned into a HindIII site of a shuttle vector.

Fig. 3 shows how the ScR-avidin-RSV cDNA was cloned into a retrovirus vector pLRNL HindIII site.

The expression of the fusion protein in cells transfected with the vector can be confirmed by Northern blotting and immunocytochemical staining with an antibody raised against avidin.

The experiments revealed that the full mRNA transcript was translated into 55 kDa monomers, which were able to form secondary structures of 110 kDa dimers attached by S-S bonds under non-reducing conditions. Approximately 110 kDa dimeric and 55 kDa monomeric peptides were detected, using denaturing conditions. The result is comparable to the computer calculation for the monomeric fusion protein, 45 kDa. In non-denaturing conditions (i.e. using acetylation prior to Western blotting), the strongest signal was approximately 220 kDa which was denatured to an approximately 110kDa dimer and a 55 kDa monomer, suggesting the formation of tetramers. The presence of the 220 kDa protein was also verified using chemical cross-linkers, e.g. NHS-esters. The results show that avidin remains soluble and is capable of forming tetramers even when attached to membrane-spanning domains of endocytotic receptors.

The fusion protein was shown to be a functional protein capable of binding FITC-biotin when analysed by confocal microscopy and atomic force microscopy. Untransduced cells and cells transfected with a retrovirus vector containing the *LacZ* gene were used as controls. No non-specific binding of biotin probes to *LacZ*-transduced control cells was detected by atomic force microscopy. As expected, the transfected cells showed specific binding that was repeatably measurable in unfixed samples. The measured binding forces were multiples of the average 149± 19pN (mean ±sd), which is, as also expected, within the range of the earlier reported biotin-streptavidin binding force of 160 pN (Florin *et al* (1994), Science 264:415-417).

Functionality of the construct can also be confirmed *in vivo* by showing the binding of fluorescently-labelled biotin molecules to cells having the fusion protein construct, using FACS analysis.

The functional activity of the fusion protein *in vivo* was analysed in a rat malignant glioma model. BT4C wild-type glioma cells were implanted intracranially in the right corpus callosum at a depth of 2.5 mm in the brain of inbred BDIX female rats. The growth of tumors was monitored frequently with high resolution MRI (magnetic resonance imaging). Three weeks after tumor cell inoculations, pseudotyped retrovirus carrying cDNA for the fusion protein or *LacZ* gene in titers of 2×10^6 cfu/ml and 1.3×10^6 cofu/ml, respectively, was transferred into the tumor, firstly at a depth of 2.5 mm and then at a depth of 1.5 mm, with a 10 minute interval. Gene transfer was repeated after two days of growth. Animals were sacrificed and perfusion-fixed with 4% PFA 3 days after the last injection. Brains were removed and divided at the injection site into two coronal pieces, sectioned on ice and analysed with immunoreactivity against anti-avidin antibody. The results showed that the fusion protein was expressed *in vivo* in rat malignant glioma. Protein was detected in glioma cells and in ring-like structures resembling vascular endothelial cells in tumor blood vessels.